Iridoid Patterns in *Galium* L. and Some Phylogenetic Considerations

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Galium, Iridoids, Phylogeny

From 19 species of *Galium*, members of 6 European sections of the genus, 24 compounds were isolated, namely 16 iridoid glucosides, 2 secoiridoid glucosides and 6 triterpene saponins (the later found only in *G. rivale* (Sibth. & Sm. Griseb.) The iridoid content was analyzed by thin layer chromatography – densitometry. An effort was made to clarify interspecies relationships, based on the obtained results and previous data. Generally, a nearly uniform iridoid pattern in the studied species was observed. Nevertheless, some distinctions gave reason the following chemical characters to be treated as taxonomic markers: iridoids, secogalioside (characteristic of *G. mollugo* group), iridoids V1 and V2 (*G. humifusum* Bieb. and *G. verum* L.), 6-acetylscandoside (*G. incurvum* group and *G. verum*) and the triterpene saponins, rivalioside A and rivalioside C (characteristic of *G. rivale*). The studied species regarding to the iridoids could be attributed to three lines of evolutionary differentiation. One line is leading to the differentiation of *G. rivale.* It contains specific triterpenoids as well as iridoid acids, which show parallel development of both glyceraldehyde 3-phosphate/pyruvate and mevalonate biosynthetic routes in this species. A second line includes *G. mollugo* and *G. incurvum* species groups and the species *G. humifusum* and *G. verum*. Variety of iridoid esters, hydroxy and carboxy derivatives of iridoids and secoiridoids characterised this line. Third line comprises the remaining studied species, members of different sections and species groups. They posses a nearly identical iridoid pattern, which suggests a convergent evolution regarding to the iridoids.

Introduction

Galium L. comprises about 400 species (Willis, 1973), 145 of which are distributed in Europe (Ehrendorfer and Krendl, 1976). In Bulgarian flora the genus is presented with 38 species (Anchev, 1992). They are characterized with considerable morphological variability, especially in the perennial representatives of the genus and interspecies hybridization, which make the species delimitation difficult.

An extensive information about morphological, karyological and ecogeographical differentiation of the genus has been accumulated (Ehrendorfer, 1971; Ehrendorfer and Krendl, 1976; Ehrendorfer and Schönbeck-Temesy, 1982; Krendl, 1987; Anchev, 1982, 1989, 1992). Ehrendorfer and Krendl's classification (1976) of the genus based on morphological, ecogeographical, palinological and karyological data has been the most widely used system.

The chemosystematic studies based on n-alkanes (Corrigan *et al*., 1978), phenols (Borisov and Zoz, 1975a,b) and iridoids (Corrigan *et al*., 1978; Inouye *et al*., 1988) are in support of some of the taxonomic decisions made by Ehrendorfer and Krendl (1976) and question others. Recently, on the grounds of enzyme and chloroplast DNA sequence analysis (Ehrendorfer *et al*., 1996; Manen *et al*., 1994; Natali *et al*., 1995, 1996) a new hypothesis on the phylogenetic relationships in the tribe *Rubieae* has been proposed.

In previous papers we reported our results on the occurrence of iridoids (Handjieva *et al*., 1996; Mitova *et al*., 1996, 1999) and triterpene saponins (De Rosa *et al*., 2000a; De Rosa *et al*., 2000b) in Bulgarian representatives of *Galium.* In this paper we analyze the iridoid patterns and discuss phylogenetic relationships among 19 species, members of 6 sections of *Galium* (Table I).

Materials and Methods

General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded at 250 and 63 MHz in CD_3OD, D_2O and pyridine-d_5

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(standards TMS and TSPA-d_4). Mass spectra were recorded on a Jeol JMS D-300 spectrometer and on a VG-ZAB mass spectrometer with a FAB source at 25 KeV (2 μA) using glycerol as matrix. Chromatography was performed on: DCCC Büchi 670 apparatus by ascending mode; HPLC on a Perkin Elmer 2/2 liquid chromatograph supplied with a Whatman ODS-3 (250 × 4.6 mm, 10 μm) column and using as mobile phase water-MeOH mixtures; low pressure chromatography: Merck Lobar RP-18 columns and water-MeOH mixtures. TLC scanning was performed on a Shimadzu CS-930 densitometer in a zigzag reflection mode with a slit of 0.4 × 0.4 mm.

Plant material

Thirty-one samples of Bulgarian natural populations of *Galium* (Table I) were collected at florescence. Nine herbarium specimens of foreign origin were kindly supplied by Dr. F. Krendl and Prof. Ih. Calis. All of the voucher specimens were deposited in the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences (SOM).

Isolation and identification of glycosides

The standards were isolated and identificated as described previously (De Rosa *et al.*, 2000a; De Rosa *et al.*, 2000b; Handjieva *et al.*, 1996; Mitova *et al.*, 1996, 1999).

Sample preparation

Dried ground aerial parts (0.4 g) were extracted with MeOH (2 × 6 ml) for 24 hours. After concentration of the combined extracts, water was added (3 ml) and threefold extraction with $CHCl_3$ (3 ml) was carried out. The water layer was threated with neutral aluminium oxide (1 g). After filtration and washing with 3 ml H_2O and 3 ml MeOH-H_2O (1:1, v/v), the combined filtrates were concentrated and dissolved in 2 ml MeOH-H_2O (1:1, v/v).

TLC analysis

Aliquots (5.0 μl) of the sample solution and 5.0 μl of the standard solution were applied to Silica gel F_{254} plates. The different mobile phases: EtOAc:iPrOH:H_2O (6:3:1) (suitable for compounds **2–4, 9, 21–22**); $CHCl_3$:MeOH:H_2O:HCOOH (75:24:1:0.2) (compounds **1, 5, 6, 8, 13, 16**) $CHCl_3$:MeOH:H_2O (60:22:4) (compounds **7, 11, 12, 14+17, 18–20, 23–24**); $CHCl_3$:MeOH:H_2O (60:15:4, lower layer) (compounds **10**, **15**) were used. Compounds were determined by scanning at 235 nm.

HPLC analysis

Gradient elution was used – pump A: H_2O-MeOH (19:1, v/v) and H_3PO_4 (15 μl per 100 ml mobile phase) and pump B: MeOH. The substances were detected at 233 nm. The flow rate was 0.8 ml/min. 10 μl of the sample solution were injected in the HPLC system.

Results and Discussion

The iridoids were chosen in this study as highly characteristic metabolites occurring mainly in the dicotyledonous plants (Dahlgren, 1989). According to Jensen (1991), for the purpose of classification, the use of biosynthetic pathways must inherently be better than using the individual compounds. This approach was used in the present study.

Plant materials (40 samples) from 31 Bulgarian and 9 foreign natural populations belonging to 19 species (Table I) were investigated. Total of 24 compounds, 16 iridoid glucosides **1–16**, two secoiridoid glucosides **17–18** (Fig. 1) and six triterpene saponins **19–24** (Fig. 2) were isolated and identified with spectral methods (1 D ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-COSY, HMQC, HMBC, MS, UV, IR) and comparison with authentic reference compounds. Biosynthetic schemes of the isolated *Galium* iridoids are represented on Fig. 1. They form *via* precursor loganic acid through geniposidic acid (**1**) and through loganin (**13**) to the further range of iridoid structures (Inouye and Uesato, 1986; Jensen, 1991; Inouye, 1991). The samples were analyzed by thin layer chromatography – densitometry and HPLC fingerprint chromatograms. The obtained data of the occurrence of iridoids and triterpene saponins in the studied samples are summarized in Table II.

Galium rivale, sect. *Trachygalium*

Galium rivale is a polymorphic species. The populations in the western and northern parts of its range differ to the southern and eastern parts in

Table I. *Galium* species investigated for iridoid glycosides and their collection localities.

No	Section/species	Voucher	Locality, m. a.s.l.	Collection date
	Sect. Aparinoides			
1	*G. palustre* L.	A 9239	Dragoman swamp, 600 m	VI.1992
2		A 9618	Rila, v. Beli Iskar, 1100 m	VI.1996
	Sect. Hylaea			
3	*G. odoratum* (L.) Scop.	A 9218	Osogovska Mt., v. Bogoslov, 1300 m	VI.1992
4		A 9282	Vitosha Mt., 950 m	VII.1992
	Sect. Trachigalium			
5	*G. rivale* (Sibth. et Sm.) Griseb.	A 9297	Struma valley, over v. Chetirzi, 500 m	VII.1992
6		A 94101	Slavjanka Mt., over v. Paril, 1200 m	VIII.1994
7[a]		31155	Slowakia, Volovske vrchy,	VIII.1995
8[a]		31156	Slowakia, Slovensky kras, 300 m	VIII.1995
9[a]		31545	Slowakia, Breziny Dubina, 580 m	VII.1996
10[a]		31544	Slowakia, Malß Tatry, 760 m	VII.1996
	Sect. Galium			
11	*G. verum* L.	A 9249	Struma valley, Kresna, 250 m	VI.1992
12		A 95156	Danube plain, Knezha, 350 m	VII.1995
13		A 9685	Stara planina Mt., Triglav, 1600 m	VII.1996
14		A 96121	Balkan foothill region, Sopot dam	VIII.1996
15	*G. humifusum Bieb.*	*A 9283*	*Danube plain, Knezha, 350 m*	*VII.1992*
16		*A 95156*	*Danube plain, Knezha, 350 m*	*VII.1995*
	Sect. Leiogalium			
17	*G. schultesii* Vest	A 9290	Znepole, v. C. Dol, 900 m	VII.1992
18	*G. pseudoaristatum* Schur	A 9289	Znepole, v. C. Dol, 950 m	VII.1992
19	*G. octonarium* (Klokov) Pobed.	A 9223	The Rhodops, Besaparski ridove, 300 m	VI.1992
	G. incurvum group			
20	*G. macedonicum* Krendl	A 9275	Struma valley, Kresna, 250 m	VII 1992
21		A 9523	Struma valley, Kresna, 250 m	VI 1995
22	*G. mirum* Rech.fil.	A 9234	The Rhodopes, Besaparski ridove, 350 m	VI 1992
23	*G. rigidifolium* Krendl	A 9474	Struma valley, Polska Skakavitza, 600 m	VII 1994
24	*G. rhodopeum* Velen.	A 9232	The Rhodopes, Besaparski ridove, 300 m	VI 1992
25	*G. aegeum* (Stoj. et Kit.) Ančev	A 94116	Slavjanka Mt., Ambar dere, 1150 m	VIII 1994
26		A 9575	Pirin Mt., v. Lovcha, 1000 m	VI 1995
27	*G. asparagifolium* Boiss. & Heldr.	A 9576	Slavjanka Mt., v. Ilinden, 900 m	VI 1995
	G. mollugo group			
28[a]	G.mollugo L.	31243	Italy, Toskana, 400–450 m	X. 1995
29[a]		30065	Rumania, Tirgu Mures, 600 m	VI. 1994
30[a]		31510	Slowakia, Štiavnické vrchy, 220 m	V. 1996
31[a]		30069	Austria, Salzburg	VIII. 1994
32	*G. lovcense* Urum.	A 9214	Konjavska Mt., l. Pazarlia, 650 m	VI. 1992
33		A 9311	Konjavska Mt., l. Pazarlia, 650 m	VI. 1993
34	*G. album* Mill. ssp. *album*	A 9286	Znepole, v. Dolno selo, 700 m	VII 1992
35		A 9480	Osogovo, v. Kolusha, 650 m	VII 1994
36	*G. album* ssp. *pychnotrichum* (H. Br.) Krendl	A 9240	Chepun, pine stands, 700 m	VI 1992
37		A 95120	Stara planina Mt., Vitinja, 750 m	VII 1995
38[b]	G. album ssp. *amanii*[b] Ehrend. et Schönb.-Tem.		Turkey, Hatay	V.1995
	Sect. Aparine			
39	*G. aparine* L.	A 9312	Struma valley, station Zemen, 600 m	VI.1993
40	*G. tricornutum* Dandy	A 9227	The Rhodopes, Besaparski ridove, 300 m	VI.1992

l. = locality; v. = village

[a] The specimens were kindly supplied by Dr. F. Krendl, Naturhistorisches Museum, Botanische Abteibung, A-1014 Wien.

[b] The specimens were kindly supplied by Prof. Ih. Çalis, Faculty of Pharmacy, Hacetteppe University, Ankara.

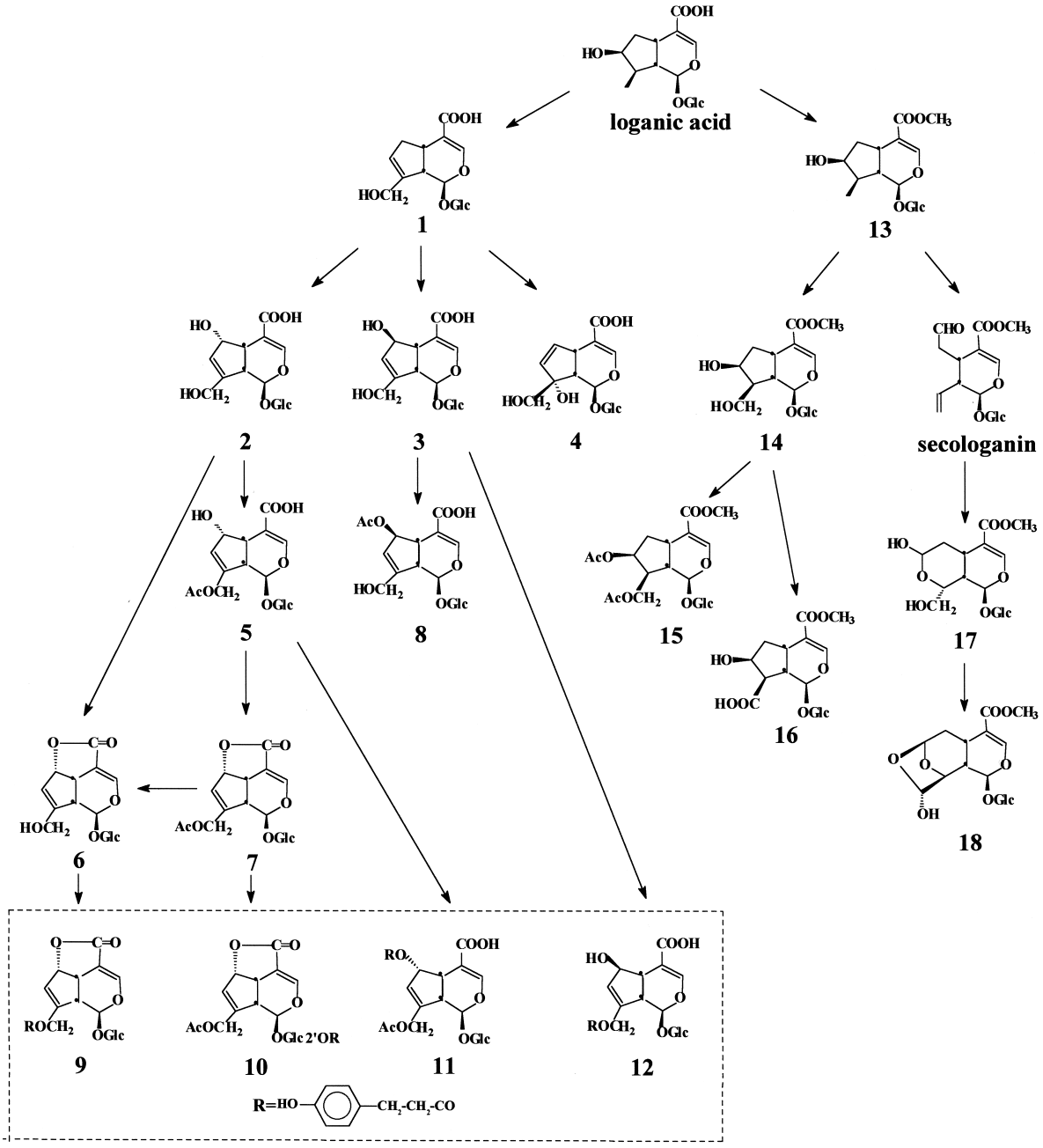
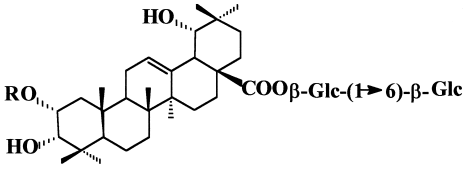
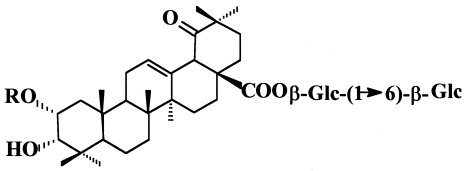


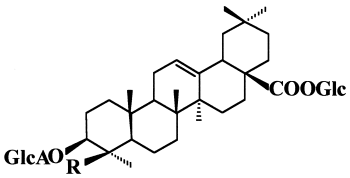
Fig. 1. Iridoids and secoiridoids isolated from the studied *Galium* species and their probable biosynthetic routes. **Compounds:** geniposidic acid (**1**), 10-deacetylasperulosidic acid (**2**), scandoside (**3**), monotropein (**4**), asperulosidic acid (**5**), deacetylasperuloside (**6**), asperuloside (**7**), 6-*O*-acetylscandoside (**8**), V1-iridoid (**9**), V2-iridoid (**10**), humifusin B (**11**), humifusin A (**12**), loganin (**13**), 10-hydroxyloganin (**14**), 7-*O*-acetyl-10-acetoxyloganin (**15**), 7β-hydroxy-11-methylforsythide (**16**), 10-hydroxymoronoside (**17**), secogalioside (**18**).



	R
Rivalioside D (19)	H
Rivalioside C (22)	Ac



	R
Rivalioside B (20)	H
Rivalioside A (21)	Ac



	R
Momordine IIb (23)	Me
Rivalioside E (24)	CH_2OH

Fig. 2. Isolated triterpene saponins from *G. rivale.*

the morphology of flowers and leaves. On this grounds Pobedimova (1958) distinguished two species (*Asperula rivalis* Sibth. & Sm., *A. aparine* L.), whereas Ehrendorfer & Krendl (1976) and Ehrendorfer & Schönbeck-Temesy (1982) did not recognize these species, because of the occurrence of transitional populations with intermediate characteristics in the Balkan Peninsula.

The poor phenols pattern of *G. rivale* separate it from the other *Galium* taxa (Borisov and Zoz, 1975a). In 19 studied *Galium* species, only in *G. rivale* we found triterpene saponins in samples from 2 localities (De Rosa *et al.*, 2000a; De Rosa *et al.*, 2000b). Another 4 herbarium specimens of a Slovakian origin (Table I: No 7–10) were additionally studied to confirm the presence of triterpenes. It was established that all these samples contained rivaliosides A (**21**) and C (**22**). Hence, the triterpene pattern is a typical character of *G. rivale* and the rivaliosides could be considered as useful chemotaxonomical markers for *G. rivale.*

The main constituents in the two investigated Bulgarian populations of *G. rivale* were the iridoid acid monotropein (**4**) and the triterpene, rivalioside A (**21**) (Table II). Recently was shown that mevalonate route is responsible for the formation of triterpenoids and glyceraldehyde 3-phosphate/pyruvate route is responsible for the formation of monoterpenoids (Rohmer, 1999). Consequently there is parallel development of the two biosynthetic routes of the terpenoids in *G. rivale.* In this species the iridoid biosynthesis is restricted to an earlier stage of formation of iridoid acids (**1–5**).

The studied two Bulgarian populations of *G. rivale* showed some qualitative and quantitative differences indicating a possibility for chemoraces. Detailed studies on a larger number of populations are required to understand the intraspecies triterpene variability and take taxonomic decisions on the base of possible morphological and/or habitat correlations (as in Pobedimova, 1958).

Galium mollugo group, sect. *Leiogalium*

This group includes *G. mollugo, G. lovcense* Urumov (=*G. protopycnotrichum* Ehrend. & Krendl), *G. heldreichii* Halacsy and *G. album* Mill. which are morphologically and ecogeographically related. In Bulgaria *G. lovcense*, *G. album* ssp. *album* and *G. album* ssp. *pycnotrichum* (H. Braun) Krendl are distributed.

The qualitative iridoid pattern supports the close relationships among the species members of

Table II. Occurrence of iridoids and triterpene saponins in the investigated *Galium* samples.

No	Taxon	Compounds																						
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14+17**	**15**	**16**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	*G.palustre*	**	**	**	**	**	tr	***	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2		**	**	**	**	**	tr	***	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	*G.odoratum*	**	**	**	***	**	*	***	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4		tr	*	*	***	*	tr	***	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	*G.rivale*	*	*	**	***	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	***	*	*	*
6		tr	*	**	***	tr	–	–	–	–	–	–	–	–	–	–	–	–	tr	tr	***	***	–	–
11	*G.verum*	–	*	**	**	**	tr	***	**	***	tr	–	–	*	–	–	–	–	–	–	–	–	–	–
12		–	*	**	**	**	tr	***	**	*	tr	–	–	*	–	–	–	–	–	–	–	–	–	–
13		–	tr	*	*	*	tr	***	*	**	tr	–	–	tr	–	–	–	–	–	–	–	–	–	–
14		–	*	**	**	**	tr	***	**	**	tr	–	–	*	–	–	–	–	–	–	–	–	–	–
15	*G. humifusum*	tr	*	**	**	*	tr	**	–	*	*	tr	tr	–	–	–	–	–	–	–	–	–	–	–
16		tr	*	**	**	*	tr	**	–	*	*	tr	tr	–	–	–	–	–	–	–	–	–	–	–
17	*G.schultesii*	*	*	*	***	*	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	*G.pseudoaristatum*	*	**	**	***	tr	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19	*G.octonarium*	tr	*	*	***	*	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20	*G. macedonicum*	–	**	**	*	**	*	***	**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
21		–	**	**	*	**	*	***	**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22	*G.mirum*	–	**	**	***	*	tr	*	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23	*G.rigidifolium*	–	**	**	**	**	tr	***	**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	*G.rhodopeum*	tr	*	*	**	*	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	*G.aegeum*	tr	**	*	**	*	tr	***	**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
26		tr	**	*	**	*	*	***	**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
27	*G.asparagifolium*	–	**	*	*	**	tr	***	**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
32	*G.lovcense*	*	**	*	**	tr	–	**	–	–	–	–	–	–	**	tr	tr	***	–	–	–	–	–	–
33		*	*	*	**	tr	–	*	–	–	–	–	–	–	*	tr	tr	**	–	–	–	–	–	–
34	*G.album* ssp *album*	*	*	**	**	*	–	*	–	–	–	–	–	–	tr	–	–	*	–	–	–	–	–	–
35		*	**	**	**	*	–	*	–	–	–	–	–	–	*	–	–	**	–	–	–	–	–	–
36	*G.album* ssp. *pychnotrichum*	*	**	*	**	*	–	*	–	–	–	–	–	–	*	–	–	***	–	–	–	–	–	–
37		*	*	*	**	*	–	*	–	–	–	–	–	–	*	–	–	***	–	–	–	–	–	–
39	*G.aparine*	tr	**	*	**	*	–	tr	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
40	*G.tricornutum*	**	*	**	**	***	*	***	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*** >1%; ** 0.5–1.0%; * 0.1–0.5%; tr <0.1%; – compound was not detected.
For the names of the compounds see Fig. 1.

the *G. mollugo* group. In the studied 31 samples belonging to 18 Bulgarian *Galium* species we found secogalioside (**18**) only in representatives of the *G. mollugo* group (Table II). Moreover, according to Bock *et al.* (1976) this compound is characteristic of *G. album* and lacks in the hybrid *G. album* × *G. verum*.

The presence of secogalioside (**18**) was proved in the additionally studied 4 herbarium specimens of *G. mollugo* of Italian, Rumanian, Slovakian and Austrian origin (Table I: No 28–31) and *G.album* ssp. *amanii* Ehrend. et Schönb.-Tem. (No 38) of Turkish origin. The areas of distribution of these taxa do not reach Bulgaria. These results give us reason to consider secogalioside (**18**) as an important chemotaxonomic marker of the *G. mollugo* group.

The iridoids **15** and **16** are present in *G. lovcense* and absent in *G. album* (Table II). Thus the qualitative iridoid pattern supports the morphological and karyological differentiation of *G. lovcense* and *G. album* and additionally proves the distinct species nature of these taxa.

G. album ssp. *pychnotrichum* and *G. album* ssp. *album* differ only in the quantitative ratio of the iridoid constituents (Table II). In *G. album* ssp. *pychnotrichum* prevails the pathway toward secogalioside (**18**), which is the main constituent of the species iridoid profile. Whereas in *G. album* ssp. *album* the pathways towards asperuloside (**7**) and secogalioside (**18**) are uniformly developed leading to a similar concentrations of asperuloside and secogalioside. This tendency was confirmed for samples from 4 different populations, which proves the subspecies differentiation. The similar qualitative iridoid pattern of the subspecies is in support of the taxonomic decision of Ehrendorfer and Krendl (1976) and Anchev (1989, 1992), op-

posite to Krendl (1987), who adopted species rank for both taxa.

The iridoids mollugoside (Iavarone *et al*., 1983), gardenosidic acid (Uesato *et al*., 1984) and its methyl ester galioside (Bianco *et al*., 1978) were isolated only from *G. mollugo*, being characteristic for this species and lack in the rest studied representatives of the *G. mollugo* group.

Galium incurvum group, sect. *Leiogalium*

The species group combines closely related narrow-leaved xerophilus species. Part of them are caespitose plants with short stems and a narrow pyramidal inflorescence (*G. rhodopeum* Velen., *G. aegeum* (Stoj. & Kitan.) Ančev, *G. asparagifolium* Boiss. et Heldr.) and others are non-caespitose plants with high stems and wide pyramidal inflorescence (*G. mirum* Rech. fil., *G. macedonicum* Krendl, *G. rigidifolium* Krendl). The iridoid 6-acetylscandoside (**8**) was found to be present in all the Bulgarian representatives of the *Galium incurvum* group exclusive of *G. rhodopeum* (Table II). However, the morphological features of the latter doubtlessly establish its position as a member of this group. Outside of the *Galium incurvum* group the iridoid **8** was found only in *G. verum* (sect.*Galium*).

The caespitose taxa of the *Galium incurvum* group produce geniposidic acid (**1**), while in the non caespitose ones geniposidic acid absent. The iridoid composition of the studied not caespitose species is identical and species chemical differentiation is not possible (Table II).

Galium verum and *Galium humifusum,* sect. *Galium*

Galium verum and Galium humifusum are morphologically well distinguishable. However, the existance of the hybrid *G. humifusum* × *G. verum* (*Galiasperula himmelbauriana* Ronn.) (Ehrendorfer and Krendl 1976; Anchev, 1989) confirms the close relations between them. The chemical profile concerning phenols and iridoids is also in support of the close relationships. Both species produce luteoline and diosmetin glycosides and only *G. verum* apigenine and kaempferol glycosides (Borisov and Zoz, 1975a,b). Only in *G. verum* and in *G. humifusum* we have found iridoid esters with p-hydroxyphenylpropionic acid like the iridoids V1 (**9**) and V2 (**10**) (Table II). Humifusin A (**12**) and humifusin B (**11**) are present in *G. humifusum* (Mitova *et al*., 1999), while loganin (**13**) and 6-acetylscandoside (**8**) are characteristic for *G. verum*.

The presence of loganin (**13**), the biosynthetic precursor for secogalioside (**18**), as well as of 6-acetylscandoside (**8**) in *G. verum,* shows the affinity of this species to the groups *G. mollugo* and *G. incurvum*. This hypothesis could be supported by some arguments. The hybridization between *G. verum* and *G. album* (Ehrendorfer and Krendl, 1976; Anchev, 1989) confirms their close relationships. The similar phenol pattern of *G. verum* and *G. mollugo* (apigenine, luteoline, diosmetine and quercitine glycosides) (Borisov and Zoz, 1975b) is another evidence. The chloroplast DNA sequence studies manifest no substantial differences between *G. verum*, 2 species of the *G. mollugo* group and 3 species of the *G. incurvum* group (Natali *et al*., 1996).

Other Galium species

The studied species *G. octonarium* (Klokov) Soo, *G. pseudoaristatum* Schur and *G. schultessii* Vest, all from sect. *Leiogalium; G. odoratum* (L.) Scop., sect. *Hylea; G. aparine* and *G. tricornutum*, both from sect. *Kolgida; G.palustre L.* from sect. *Aparinoides,* are mesophytes and hygrophytes, except for a single xerophyte, namely *G. octonarium*. According to the chloroplast DNA sequence studies the representatives of sectt. *Leiogalium*, *Hylea* and *Kolgida* belong to one clade, as *G. odoratum* and studied sect. *Kolgida* species form a subgroup (Manen *et al*., 1994; Natali *et al*., 1995, 1996). The research of Borisov and Zoz (1975a,b) showed that *G. odoratum* (sect. *Hylea*) and the representatives of sect. *Kolgida* possess a similar phenol pattern (phenol acids, depsides, flavonol glycosides and a lack of flavan glycosides). Our study of the iridoids of the above-mentioned *Galium* species demonstrates that they have almost identical qualitative iridoid patterns (Table II). The similar iridoid and phenol pattern and similar chloroplast DNA sequence could be explained by convergent evolution. However, the affinity between *G. odoratum* (sect. *Hylea*) and sect. *Kolgida* must not be excluded.

G. palustre, a member of the sect. *Aparinoides*, which contains hygrophylus plants with different

basic chromosome number (Ehrendorfer & Puff, 1976) and a specific chloroplast DNA sequence (Manen *et al*., 1994; Natali *et al*., 1995, 1996), has an iridoid content identical to that found in the studied representatives of sectt. *Leiogalium*, *Hylea* and *Kolgida*. In this case, convergent evolution regarding iridoids of *G. palustre* is doubtless.

Phylogenesis of the studied Galium species

The obtained data show that iridoid acids **2**, **3**, **4** and **5** are characteristic for all studied species (Table II). It suggests that these compounds have evolved early in their common ancestry. Regarding to the iridoids, the phylogenesis of ancient ancestors of the studied *Galium* species went in three different ways.

An evolutionary line led to the differentiation of *G. rivale*. It is characterized with a paralell development of both mevalonate and glyceraldehyde 3-phosphate/pyruvate routes. The iridoid biosynthesis is restricted to an earlier stage of formation of iridoid acids (**2–5**). *G. rivale* is known only with hexaploid populations, which suppose an ancient origin of this species. These data are in support of an earlier differentiation of *G. rivale* under different habitat conditions where evolutionary "experiments" with different defense compounds of terpenoid nature were developed.

All species of the other two lines characterised with presence of asperuloside (**7**) (Table II), which is biosynthetized at a later stage (Fig. 1). Therefore, at the beginning they had common phylogenesis regarding to iridoids.

The line including *G. mollugo* and *G. incurvum* species groups, *G. humifusum* and *G. verum* is characterised by variety of iridoid esters, hydroxy and carboxy derivatives of iridoids and secoiridoids. The data suppose that above-mentioned species are closely related and consequently have common ancestor. Obviously the ancestor of this phylogenetic line persisted at environmental conditions where different types of iridoids gave competitive advantage and stimulated branching of the iridoid biosynthetic routes.

A third line comprises the remaining studied representatives of the genus. Morphologically these species are well differentiated, but they posses a nearly identical iridoid pattern. Evidently the morphological evolution of these species was divergent but evolution regarding to the iridoids and some other characters were convergent. The habitat conditions did not stimulated the considerable branching of the iridoid biosynthetic routes, nor yet the developing of the other terpenoids biosynthetic routes.

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